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EXHIBIT A (2/4)

Animal Model

Impaired Hair Follicle Morphogenesis and Cycling with Abnormal Epidermal Differentiation in *nackt* Mice, a Cathepsin L-Deficient Mutation

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We previously described an autosomal-recessive mutation named *nackt* (*nkt*) exhibiting partial alopecia associated with CD4$^+$ T-cell deficiency. Also, we recently reported that *nkt* (now *Ctsl*^{nkt}) comprises a deletion in the cathepsin L (*Ctsl*) gene. Another recent study reported that *Ctsl* knockout mice have CD4$^+$ T-cell deficiency and periodic shedding of hair, which recapitulate the *nkt* mutation and the old *furless* (*fs*) mutation. The current study focuses on the dermatological aspects of the *nkt* mutation. Careful histological analysis of skin development of homozygous *nkt* mice revealed a delayed hair follicle morphogenesis and late onset of the first catagen stage. The skin of *Ctsl*^{nkt}/*Ctsl*^{nkt} mice showed mild epidermal hyperplasia and hyperkeratosis, severe hyperplasia of the sebaceous glands, and structural alterations of hair follicles. Epidermal differentiation seems to be affected in *nkt* skin, with overexpression of involucrin and profilaggrin/filaggrin along with focal areas of keratin 6 expression in the interfollicular epidermis. Severe epidermal hyperplasia, acanthosis, orthokeratosis, and hyperkeratosis were only observed in mice maintained in nonpathogen-free environments. The analysis of *Rag2*−/− *Ctsl*^{nkt}/*Ctsl*^{nkt} double-mutant mice indicates that the skin defect remains under the absence of T and B cells. This animal model provides *in vivo* evidence that cysteine protease cathepsin L plays a critical role in hair follicle morphogenesis and cycling, as well as epidermal differentiation. (*Am J Pathol 2002, 161:693–703*)

The molecular mechanisms that control epidermal differentiation, hair follicle (HF) morphogenesis, and its cyclic transformation in mammals are not yet fully understood. In this regard, the availability of many transgenic, knockouts, and spontaneous mutant mice exhibiting hair growth and skin defects has been pivotal in the understanding of HF and epidermal biology.[1–3] The autosomal-recessive mutation *nackt* (*nkt*) shows a delay in the appearance of the first pelage and a sparse hair coat with partial alopecia (focalized on the back) throughout life, as well as CD4$^+$ T-cell deficiency. The *nkt* trait has 100% penetrance and no genetic modifiers were detected affecting expressivity or pleiotropy, because no phenotype modifications were observed in different genetic backgrounds, including wild-derived inbred mice.[4,5] The *nkt* mutation comprises a deletion in the cathepsin L (*Ctsl*) gene (a lysosomal cysteine protease) with no functional protein present, rendering the mutation a spontaneous functional knockout for the *Ctsl* gene. Because the mutated gene is now known, the complete gene symbol is *Ctsl*^{nkt}. We mapped the mutant allele to mouse chromosome 13 (37 cM) and in that way refined the mapping of the *Ctsl* gene.[5] Mouse *Ctsl* gene mapping had been previously reported by Pilz and collaborators[6] based on specific backcross linkage analysis, and later by Deussing and colleagues[7] using a panel of BXD recombinant inbred strains. The *Ctsl* locus is located in a region of mouse chromosome 13 that shows synteny homology with a region on human chromosome 9. The human *CTSL* gene has been localized in this conserved syntenic region at 9q21-q22.[8]

Cathepsins are a family of lysosomal proteases with aspartyl and cysteinyl members that are believed to be involved in extracellular and intracellular protein degradation as well as in a range of cellular processes such as

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major histocompatibility complex antigen presentation and bone remodeling.[9] There are currently two known aspartyl (cathepsins D and E) and 11 known cysteinyl (cathepsins B, H, L, S, C, K, O, F, V, X, and W) lysosomal proteases. Cathepsins L (CTSL) and B (CTSB) are, together with aspartyl protease cathepsin D (CTSD), the most abundant lysosomal proteases. Using *Ctsl* knockout mice, Nakagawa and collaborators[10] recently found that CTSL is critical for degradation of invariant chain, a critical chaperone for major histocompatibility complex class II molecules, in cortical thymic epithelial cells. In addition, *Ctsl* knockout mice experience CD4 deficiency and periodic shedding of hair with abnormal skin morphology, a phenotype that is reminiscent of the *nkt* and *fs* mutants.[11] More recently, Roth and colleagues[12] have described the deficiency of CTSL as the molecular defect underlining the *fs* (a loss of function mutation of *Ctsl*) and *Ctsl−/−* mouse skin phenotypes. Thus, CTSL was the first lysosomal proteinase to be implicated as an essential component of the epidermal homeostasis and HF morphogenesis and cycling.

So far, all of the studies comprising *Ctsl*^{nk1}/*Ctsl*^{nk1} mice (hereafter abbreviated *nkt/nkt*) have been concentrated on the CD4$^+$ T-cell deficiency. Thus, the mechanism of the hair loss and cycling defects in *nkt/nkt* mice has never been described. The dermatological aspects of the *nkt* mutation presented here are an initial effort to address the questions regarding the role of CTSL in HF morphogenesis and cycling, as well as epidermal differentiation in the *nkt* skin. Although reminiscent of the *Ctsl−/−* phenotype, the *nkt* mice present unique characteristics, such as the hyperplasia of sebaceous glands, which can be attributed to the nature of the mutation. We present, for the first time, a thorough description of the skin phenotype using various and well-defined genetic backgrounds on a specific pathogen-free (SPF) as well as conventional (nonpathogen-free) environment. We used a genetic approach to prove that the skin phenotype is the direct effect of a deficiency of CTSL on the skin and not an epiphenomenon of the immunological phenotype. Our observations from the *nkt* skin suggest a role of CTSL in the terminal differentiation of keratinocytes in the epidermis, as well as HF morphogenesis and cycling. The processing of profilaggrin to filaggrin and the final proteolytic steps of filaggrin degradation seems to be impaired in the *nkt* skin, leading to a delay in transit from stratum granulosum to stratum corneum. This mouse mutation may provide a good model for the study of human disorders involving alopecia, sebaceous gland hyperplasia, hyperkeratosis, and defective differentiation of the epidermis.

Materials and Methods

Animal Model and Skin Samples

The *nkt* mutant allele arose in 1981, in the laboratory animal facilities of the Martin-Luther-Universität in Halle-Wittenberg (Germany). A pair of mutant mice was introduced into the animal facility of the Institut Pasteur, Paris, France, in 1991, where they were crossed to 129S2/SvPas inbred breeders for a few generations and then intercrossed. Mice segregating for *nkt* were transferred to the animal facilities of the Instituto de Investigaciones Hematológicas, Buenos Aires, Argentina, where the mutant allele was backcrossed onto two new backgrounds (BALB/c and C57BL/6) by performing, in each case, four successive rounds of cross-intercross (N4). Finally, these N4 mice segregating for *nkt* were transferred to the M. D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX, where we continued backcrossing onto BALB/c and C57BL/6 backgrounds (currently in N9). (Nomenclature for these congenic strains is BALB/c.Cg-*Ctsl*^{nk1} and C57BL/6.Cg-*Ctsl*^{nk1}). We have also collected data for the description of the gross appearance of *nkt* animals from MAI/Pas, MBT/Pas, and CAST/Ei wild-derived inbred strains (used in the genetic mapping project) and partially congenic DBA/2-*nkt* (N4) mice. BALB/c-*Rag2−/−* (C.129S6(B6)-*Rag2*^{tm1}) congenic mice were purchased from Taconic Farms (Germantown, NY).

For studies of HF morphogenesis and cycling, groups of three male and three female mice at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 days postpartum from *nkt/nkt* and *+/nkt*? littermates (BALB/c and C57BL/6 backgrounds) were used, to cover the first hair cycle.[13] Skin specimens were harvested from these mice as well as from adult mice at 6 weeks and 6 months of age, including homozygous and heterozygous mutant animals as well as wild-type. Skin collection was also done at 2, 3, and 6 weeks of age from BALB/c-*Rag2−/−*; *nkt/nkt* double-mutant mice. Animals were sacrificed by CO_2 asphyxiation and skin samples from the dorsum, ventrum, muzzle, ear, eyelid, footpad, and tail were collected in Fekete's acid-alcohol formalin fixative. Within 12 hours of collection the samples were fixed in 70% ethanol, then embedded, sectioned, and processed for hematoxylin and eosin (H&E) and Giemsa staining as described by Sundberg and Boggess.[14] Heads, as well as front and rear feet, whole mounts were previously decalcified. For hair studies, hair samples were plucked (truncal hair) from around the alopecic areas and normal areas and examined by light microscopy. Complete sets of organs were collected, fixed, processed, and stained with H&E for histopathological examination from mice at the referred ages. The stages of HF morphogenesis and cycling were assessed according to Paus and colleagues[15] and Sundberg and King,[13] respectively, in 50 HFs per animal.

Bromodeoxyuridine (BrdU) Labeling

Epithelial cell proliferation was measured by intraperitoneal injection of BrdU (60 μg/g; Sigma Chemical Co., St. Louis, MO) 30 minutes before the mice were sacrificed. Dorsal and ventral skin from both mutant and control animals were collected and fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. After sectioning, the slides were dewaxed and denatured in 1.5 mol/L of HCl for 30 minutes and neutralized with phosphate-buffered saline for 1 hour. BrdU incorporation was detected by immunohistochemical staining of paraffin-

embedded sections with mouse anti-BrdU monoclonal antibody (Becton-Dickinson Immunocytometry System, Becton-Dickinson, San Jose, CA). The reaction was visualized with a biotin-conjugated anti-mouse antibody (Vector Laboratories, Inc., Burlingame, CA) and avidin-biotin-peroxidase kit (Vectastain Elite, Vector Laboratories, Inc.) with diaminobenzidine as chromogen, as previously described.[16]

Immunohistochemistry

Dorsal skin from *nkt/nkt* and *+/nkt?* control mice was fixed in 70% ethanol or snap-frozen (embedded in OCT) in liquid nitrogen for immunohistochemistry immediately after the mice were sacrificed. Longitudinal cryosections (8-μm thick) were fixed in acetone for 10 minutes at −20°C and air-dried at room temperature for 6 hours. Immunohistochemical staining of ethanol-fixed paraffin-embedded tissues was performed with polyclonal antibodies directed against mouse keratins K1, K5, K6, K10, K14, loricrin, involucrin, and profilaggrin and filaggrin (Covance Research Products, Richmond, CA), and Cd31 (PharMingen, San Diego, CA) as previously described.[17–20] We assessed standard microvessel density with the anti-Cd31 (platelet/endothelial cell adhesion molecule) monoclonal antibody as previously described.[21] Controls in the absence of primary antibodies were routinely performed. To determine whether possible changes in expression of these epidermal proteins were solely because of the epidermal hyperplasia displayed by the *nkt* skin, we included in our studies 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal hyperplasias. The samples used for the hyperplastic skin analysis were a gift from Dr. I. B. Gimenez-Conti. For this purpose, SENCAR mice were treated topically twice a week with 5 g of TPA (Sigma Chemical Co.) per animal.

Western Blotting

Protein was isolated from separated dorsal epidermis of *nkt* mutants and control mice using RIPA lysis buffer (150 mmol/L NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mmol/L Tris, pH 8.0) and run on a 10% Tris-glycine gel obtained from Novex (San Diego, CA). Protein concentration was estimated using the Bradford reagent obtained from Sigma (The Woodlands, TX) and an Eppendorf Biophotometer (Brinkmann, Westbury, NY). Polyclonal rabbit anti-mouse keratins K1, K5, K6, K10, K14, loricrin, involucrin, and profilaggrin and filaggrin antibodies were obtained from Covance Research Products. Binding of antibodies was detected using ECL+Plus Western blotting detection system (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK). We also included TPA-induced epidermal hyperplasia in the Western blot studies. Protein extracts were a gift of Dr. J. DiGiovanni (Science Park Research Division, M.D. Anderson Cancer Center, Smithville, TX) (obtained from whole skin of FVB/N mice with four treatments of 3.4 nmol of TPA throughout 2 weeks).

Results

Gross Observation and Clinical Features

From the time of birth until approximately 7 days of age, mutant *nkt* mice appeared runted in comparison with littermate controls (Figure 1A). At postnatal days 5 to 6, the skin of heterozygous littermates showed the first pelage hairs emerging through the epidermis, whereas homozygous mutants remained completely naked. The first hair coat in *nkt/nkt* mice developed at approximately day 7 of age and continued to be sparse during the entire life span of the animals. In contrast to *Ctsl−/−* and *fs/fs* mice, described by Roth and collaborators,[12] mutant *nkt* mice never show a normal, healthy fur. Figure 1B shows the sparse coat phenotype of a 15-day-old BALB/c-*nkt/nkt* animal. The gross appearance of adult *nkt* mice was always associated with focal alopecia and completely bald patches around the eyes and the cranial region of the dorsum (Figure 1C). Homozygous *nkt* mice have normal vibrissae, eyelids, and nails. Heterozygous *nkt* mice were indistinguishable from wild-type age-matched mice at all ages.

When maintained in non-SPF facilities (albeit free of ectoparasites), *nkt/nkt* mice were nearly devoid of hair at 3 weeks of age. By 3 months of age, the mutant mice were severely pruritic based on intense scratching by the animals, suggesting the association of bacterial or fungal infections. This often resulted in self-inflicted lesions that evolved into dermatitis and ulceration (Figure 1D). In older animals, these lesions were always associated with enlargement of the regional lymph nodes (especially of the head, neck, and hind limb regions). This dermatopathic lymphadenitis was associated with the generalized exfoliative dermatitis originated from the chronic pruritus.

HF Morphogenesis and Cycling

Histological examination of the skin revealed differences between homozygous and control mice (maintained in SPF conditions) in the morphology of the developing HFs. Day 6 homozygous *nkt* mice showed a delay in the morphogenesis compared with littermate controls, confirming the macroscopic observation. Following the guidelines proposed by Paus and colleagues,[15] we determined that 80% of the mutant HFs were on stage 6 whereas 60% of wild-type HFs were clearly at stage 8 (Figure 2, A and B). Also, the onset of the first catagen cycle was delayed in day 21 postpartum *nkt/nkt* mice. Approximately 60% of HFs in wild-type back skin had already reached the end of catagen of the first hair cycle. In contrast, only 30% of *nkt/nkt* follicles had completed catagen development (Figure 2, C and D). Also, homozygous mutant mice exhibited a reduced HF density (data not shown).

Microscopic Description

At approximately day 15 postpartum, the presence of large sebaceous glands in homozygous compared to

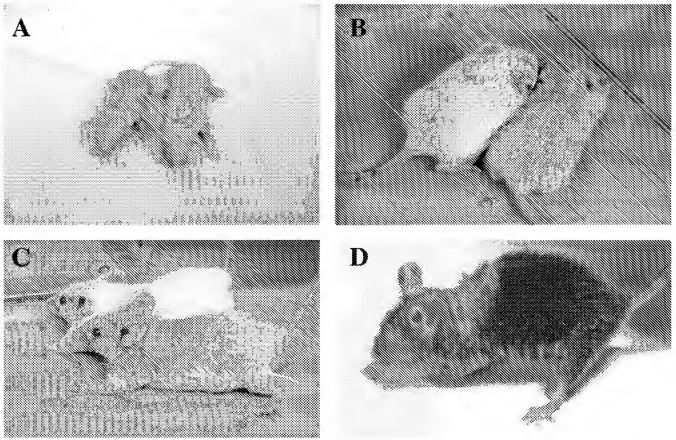

Figure 1. Skin phenotype of *Ctsl*^{nkt}/*Ctsl*^{nkt} mice. **A:** BALB/c-*Ctsl*^{nkt}/*Ctsl*^{nkt} mouse (**left**) is runted at day 3 postpartum compared with age- and sex-matched littermate controls (**right**). The first hair coat in mutant mice develops at approximately day 7 postpartum and continues to be sparse during the entire life span of the animals. **B:** Sparse coat phenotype of a BALB/c-*Ctsl*^{nkt}/*Ctsl*^{nkt} mouse (**right**) at day 15 postpartum. **C:** Focal alopecia of the cranial region of the dorsum in a BALB/c-*Ctsl*^{nkt}/*Ctsl*^{nkt} mouse (**front**) at day 90 postpartum. **D:** Self-inflicted lesions and skin ulcerations in a 9-month old MAI/Pas-*Ctsl*^{nkt}/*Ctsl*^{nkt} mouse maintained in non-SPF environment.

control mice (maintained in SPF conditions) was evident (Figure 2, E and F). This is a prominent feature that was not described in the skin of *fs/fs* and *Ctsl−/−* mutant mice.[12] Also beginning at approximately day 15, the epidermis of *nkt* mutant mice was moderately hyperplastic and mildly acanthotic and showed various degrees of orthokeratotic hyperkeratosis with the presence of scattered keratin plugs. However, epithelial cell proliferation measured by BrdU labeling did not reveal significant differences between mutant and wild type (data not shown). Standard microvessel density measured with anti-Cd31 monoclonal antibody did not reveal significant differences between mutant and control mice (data not shown). Scant numbers of HFs, often devoid of hair shafts and moderately atrophic, were also observed in the *nkt* skin. Hair shafts were defective (often twisted) with dilation of the hair canal and showed occasional adherent keratinous debris. Mutant *nkt* mice seemed to have a propensity for follicular accumulation of keratin. External ear canals were filled with excess keratin, and the nonglandular stomach showed mild hyperplasia with orthokeratosis. The dermis of SPF homozygous mutant mice appeared slightly thickened and showed long stretches without HFs (Figure 2C). Giemsa staining of mast cells did not reveal significant differences between mutant and control mice (data not shown).

Histologically, the epidermis of mutant mice maintained in non-SPF (conventional) conditions appeared severely hyperplastic with acanthosis and/or hypergranulosis, or epidermal and follicular orthokeratosis. The propensity for follicular and sebaceous gland accumulation of keratin sometimes led to furunculosis and abscesses of the preputial glands. Multifocal areas of sercocellular crust formation were observed on the skin of older animals. The dermis showed marked infiltration of inflammatory cells such as lymphocytes, mast cells, and polymorphonuclear cells. There was also marked hyperplasia of the sebaceous gland, which was presumably enhanced as a consequence of the dermatitis. Skin lesions were consistently less severe in the ventral region than in other parts of the body. Homozygous *nkt* mice frequently developed opacification of the cornea with a rather heterogeneous phenotype along with keratitis and ulceration. In aged *nkt* mice (approximately 1 year old), multifocal areas of necrosis were found in the periodontal ligament and pulp cavity of molars, along with cementum resorption.

Keratin Expression

To further investigate the mechanisms involved in the defective epidermal differentiation of the *nkt* skin, we

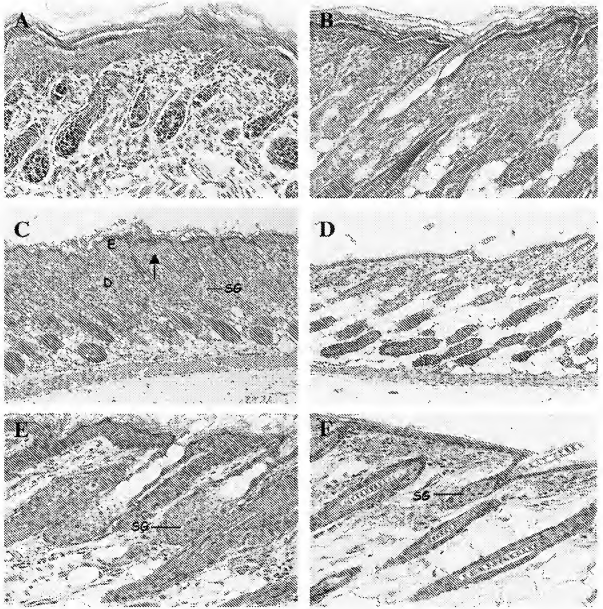

Figure 2. Delayed HF morphogenesis and sebaceous gland hyperplasia in BALB/*c-Ctsl*^{nkt}/*Ctsl*^{nkt} mice. **A:** Histological analysis of day 6 postpartum sections of mutant back skin shows that most of the HFs are on stage 6 of morphogenesis.[15] **B:** Skin sections of age- and sex-matched littermate controls show HFs at stage 8 (hair shaft emerging through the epidermis) (H&E; original magnifications, ×200). **C:** Day 21 postpartum sections of mutant back skin show a delay in catagen progression compared with age- and sex-matched littermate controls (**D**). Also note the presence of hyperplastic epidermis (**arrow**) and thickened dermis in homozygous compared to control mice (H&E, original magnifications, ×100). **E:** Marked hyperplasia of sebaceous gland in day 15 postpartum mutant mice compared with age- and sex-matched littermate controls (**F**) (H&E; original magnifications, ×200). E, epidermis; D, dermis; SG, sebaceous gland.

performed immunohistochemistry with antibodies against K1, K5, K6, K10, and K14.[22] Immunohistochemical analysis of 21-day-old *nkt/nkt* back skin with markers against K1, K5, K10, and K14 showed no significant differences between *nkt/nkt* mice and littermate controls. We found that in back skin from 21-day-old *nkt/nkt* mice, K6 expression is present in the HFs and also in the interfollicular epidermis as focal areas around the HF. As shown in Figure 3A, *nkt/nkt* skin exhibits K6-positive keratinocytes emerging from the HF to the epidermis, a feature not found in age- and sex-matched littermate controls. In normal untraumatized skin, K6 expression is restricted to specialized cells of the HF, particularly a single cell layer that surrounds the innermost layer of the outer root sheath. However, inducible expression in the interfollicular epidermis takes place in response to stressful stimuli or exposure of phorbol esters.[23] This feature of the *nkt* skin implies altered epidermal differentiation and suggests that the re-epithelialization process of the mutant hyperplastic epidermis originates from the HF keratinocytes. By Western blotting, we could confirm that expression of K6 was higher in the *nkt* skin, compared to TPA-induced hyperplastic skin. However, we found no significant differences between *nkt/nkt* mice and littermate controls in the expression of K1, K5, K10, and K14 (Figure 4).

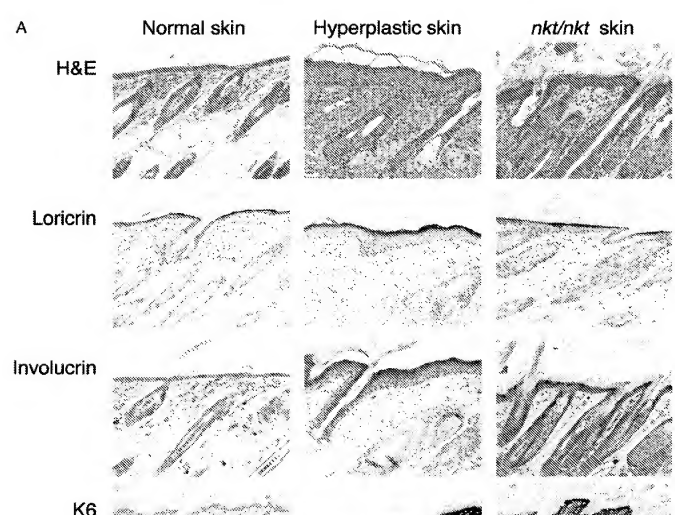

Figure 3. Histology and immunohistochemistry of BALB/c-*Cts*l^{nkt}/*Cts*l^{nkt} mouse epidermis. H&E staining and immunohistochemistry of normal (BALB/c-+/*nkt*?), hyperplastic (TPA-treated SENCAR mice), and *nackt* (BALB/c-*nkt*/*nkt*) dorsal skin. **A:** Note high levels of involucrin expression in the epidermis and K6 expression in the interfollicular epidermis in *nkt*/*nkt* skin. **B:** Note the marked increase of profilaggrin and filaggrin expression in *nkt*/*nkt* epidermis. Original magnifications: ×200, except profilaggrin and filaggrin ×200 and ×400. Immunohistochemistry was performed with loricrin, involucrin, K6, K10, K14, and profilaggrin and filaggrin antibodies

Involucrin, Loricrin, and Profilaggrin/Filaggrin Expression

A panel of antibodies was used to evaluate, by immunohistochemistry, the expression of mouse-specific epidermal involucrin, loricrin, and profilaggrin/filaggrin in mutant and control mice. Involucrin was weakly positive in the upper epidermis in normal skin; however, in *nkt/nkt* mice, the epidermis exhibited a marked positive signal with a diffuse staining of the lower layers and sebaceous gland. TPA-induced hyperplastic epidermis also showed a strong signal in most of the suprabasal layers as previously reported (Figure 3A).[24] Late marker loricrin was expressed in similar patterns in both mutant and control mice, but was slightly overexpressed in the TPA-induced hyperplastic epidermis (Figure 3A). Profilaggrin and filaggrin were expressed in keratohyaline granules in normal skin and TPA-induced hyperplastic skin, but the signal was more prominent in the latter (Figure 3B). In *nkt/nkt* mice, profilaggrin/filaggrin were strongly overexpressed on the granular layer and showed a diffuse staining in most of the suprabasal layers of the epidermis and in the sebaceous glands (Figure 3B). To determine this further, immunoblot analysis was performed using epidermal extracts prepared from dorsal skins of normal (+/+ and +/*nkt*), TPA-treated, and *nkt/nkt* mice. Involucrin was

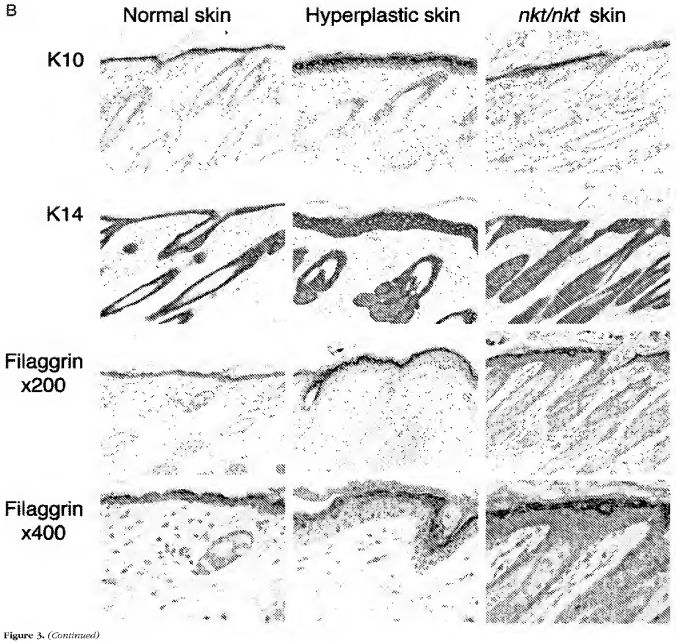

Figure 3. *(Continued)*

overexpressed at high levels in *nkt/nkt* mice, compared to normal and even hyperplastic skin (Figure 4). Further, the *nkt* epidermis showed a significant increase in profilaggrin, filaggrin intermediates, and filaggrin, suggesting a defect in the processing of profilaggrin to filaggrin and the final proteolytic steps of filaggrin degradation (Figure 4).

Rag2−/− Ctsl^{nkt}*/Ctsl*^{nkt} *Double-Mutant Mice Retain the Skin Phenotype*

HF cycling in the mouse was previously associated with alterations in the skin immune status, particularly in the number of mast cells, macrophages, and T cells.[25] Also, infiltration of lymphocytes in the HF can follow with disruption of its normal function and give rise to defective hair shafts (albeit without hyperplastic epidermis) as was reported in aging C3H/He mice.[26] To address the question of the involvement of immunological alterations as possibly being responsible for the skin phenotype of the *nkt* mice, we developed double mutants harboring a *Rag2* null allele and the *nkt* allele. Gross as well as histological examination of the skin at 2, 3, and 6 weeks of age revealed no differences between homozygous *nkt/nkt* and *Rag2−/− nkt/nkt* double-mutant mice (data not shown). This finding demonstrates that the skin defect in the *nkt* mutant mice is not a lymphocyte-mediated process but a direct effect of the deficiency of CTSL in the skin.

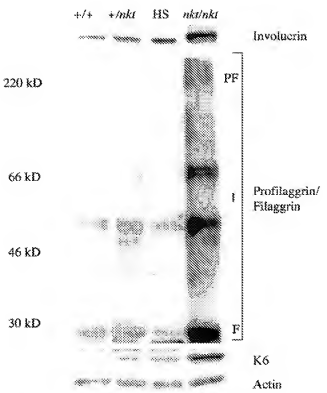

Figure 4. BALB/c-*Ctsl*^{nkt}/*Ctsl*^{nkt} epidermis expresses high levels of involucrin, K6, and profilaggrin/filaggrin. Western blot analyses were done with normal (+/+ and +/*nkt*) and *nkt/nkt* epidermis from day 21 postpartum mice, and hyperplastic skin (HS) from adult FVB/N mice treated with TPA. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. Note overexpression of involucrin and K6 as well as the marked overexpression of high molecular weight profilaggrin (PF), filaggrin (F), and its processing intermediates (I) in *nkt/nkt* epidermis. Filaggrin (27 kd in the mouse) is synthesized as a high molecular weight precursor, profilaggrin (>200 kd), which is subsequently processed. The *nkt/nkt* epidermis seems to have a defect in the processing of profilaggrin to filaggrin and its final proteolytic degradation. Western blot was performed with involucrin, K6, profilaggrin/filaggrin, and actin (loading control) primary antibodies as described in Materials and Methods.

Discussion

Various mouse mutations with anomalies of the hair coat and associated with immunological defects have been previously reported. This is the case for the nude locus (*Foxn1*^{nu} and *Foxn1*$^{nu\text{-}slf}$), in which affected mice are hairless from birth and suffer from almost complete thymic agenesis.[27] The pleiotropic effect of this mutation is explained by the disruption of the *Foxn1* gene, a transcription factor expressed specifically in both skin and thymus and required for the proper keratinization of the hair shaft.[28] The balding allele (*Dsg3*^{bal}), a frameshift mutation of the *Desmoglein 3* gene, an adhesion molecule, likewise exhibits both hair growth abnormalities and an increase in the number of mast cells in skin with associated elevated IgE levels.[29,30] The flaky skin (*fsn*) mutation, a potential model for human psoriasis, has also elevated serum IgE levels and mast cell accumulation associated with lymphadenopathy.[31] Finally, hairless mice (*hr* and *hr*^{n} alleles), which lose their coat after the first hair pelage, have a defective cellular immune response throughout their life. A transcription factor defect irreversibly abrogates the follicle's capacity to cycle in *hr/hr* mice.[32] These spontaneous mouse mutations, among many others, have been critical in the comprehension of the developmental biology of the HF.[2,33,34]

We report here the dermatological characteristics of a spontaneous mouse mutation that shows partial alopecia, reduced HF density, and sebaceous gland hyperplasia associated with abnormal epidermal differentiation. This immunodeficient mutation comprises a deletion in the *Ctsl* gene, a lysosomal cysteine proteinase.[4,5] Although the *nkt* mouse does not represent a model for a particular inherited human skin disease, mutant mice exhibit morphological similarities that correlate with different human disorders and offer a potential model to study their biochemical mechanisms (Table 1). The potential use of experimentally infected *nkt* mice as a model for the study of dermatitis associated with infections in immunodeficient individuals (eg, HIV-associated dermatitis) remains to be evaluated.

Papain-like cysteine proteinases have long been associated with terminal degradation of proteins in the lysosomal compartment. However, recent findings have highlighted the existence of more specific functions of these enzymes, such as antigen processing and presentation, osteoclastic bone resorption, and cancer invasion.[35] In particular, CTSL has been shown to be essential for antigen processing in cortical thymic epithelial cells as well as epidermal homeostasis and HF morphogenesis and cycling.[10,12,36] In the skin, catagen was previously associated with extracellular matrix remodeling and condensation of the dermal papilla, and intrafollicular and perifollicular protease-anti-protease systems have been implicated in the control of this remodeling process.[37,38] Involvement of lysosomal proteolysis-associated macrophages in the sequence of morphological and pigmentary changes during the anagen to catagen transforma-

Table 1. Comparison of Features of *nkt/nkt* Mice and Human Skin Disorders

Characteristics in *nkt/nkt* mice	Human disorder
CTSL deficiency	PLS and HMS
(autosomal-recessive mutation)	(CTSC deficiency, autosomal recessive trait)
Acantosis and hyperkeratosis associated with periodontitis in non-SPF mice	PLS and HMS
Hyperkeratosis associated with increased K6, invilucrin, and filaggrin expression	BCIE
Hyperplasia of sebaceous glands	NSJ and Senile sebaceous hyperplasia

PLS, Papillon-Lefèvre syndrome; HMS, Hain-Munk syndrome; BCIE, bullous congenital ichthyosiform erythroderma or epidermolytic hyperkeratosis; NSJ, nevus sebaceus of Jadassohn.

tion was also suggested.[25,39] A number of different proteases have been shown to play a role in epidermal differentiation, including calpain I and cathepsins B, C, H, and L.[40] Aspartic protease CTSD was proposed to play a role as a proteolytic agent of desquamation in human skin.[41] Furthermore, activated forms of CTSL, along with CTSB and CTSD, were proposed to play a role in the pathogenesis of psoriatic epidermis.[42] Roth and collaborators[12] postulated that in the absence of CTSL, the skin phenotype of *Ctsl−/−* mice is because of hyperproliferation of HF epithelial cells and basal keratinocytes. In addition, using CTSL-deficient fibroblasts from *Ctsl−/−* mice, Zwad and collaborators[43] showed transient accumulation of insulin-like growth factor-1 binding protein-3 in the endosomal and lysosomal compartments. This observation suggested that CTSL might play a role in the balance between proliferation and differentiation of epithelial cells through insulin-like growth factor-1.

During terminal differentiation of keratinocytes, profilaggrin (found in keratohyalin granules) is processed to functional filaggrin, which binds to keratin filaments.[44,45] Recently, it was proposed that filaggrin is susceptible to degradation by an epidermal CTSL-like proteinase in the rat, suggesting that this proteolytic activity may have relevance to skin differentiation.[46] By immunohistochemistry, we showed that K1, K10, K14, and loricrin expression follow predictable patterns in hyperplastic skin, with similar patterns in the hyperplastic epidermis of *nkt* mice, namely, increase in K14 expression, results confirmed by Western analysis. However, expression of K6 in the interfollicular epidermis, increased levels of involucrin, and strong overexpression of filaggrin was observed only in the *nkt* skin by immunohistochemistry. By Western blotting, we confirm an increase in K6 and involucrin expression as well as a significant increase in profilaggrin, filaggrin intermediates, and filaggrin in the *nkt* epidermis. Our results suggest that CTSL is playing a role in the conversion of profilaggrin to filaggrin as well as the degradation of filaggrin to free amino acids in the stratum corneum during keratinization. The increase in the number of cells expressing filaggrin in the upper layers of the epidermis suggests a delay in the transit of filaggrin from the stratum granulosum to the stratum corneum. However, the mild epidermal hyperplasia seems to be the result of a disturbed program of epidermal differentiation rather than an increase in the proliferation rate, because the total number of proliferating cells (as measured by BrdU labeling) in the *nkt* skin was comparable to control mice. This is supported by previous evidence that expression of K6 is not directly linked to hyperproliferation but indirectly to altered epidermal differentiation.[23] Thus, the hyperkeratosis may result from a decelerated desquamation rather than an accelerated cornification process. Similar increases in profilaggrin, filaggrin intermediates, and filaggrin were reported in human patients with epidermolytic hyperkeratosis (also called bullous congenital ichthyosiform erythroderma or BCIE).[47] Increases in involucrin, filaggrin, and K6 have been reported also in K10-deficient mice, an animal model for human BCIE.[48,49]

Recently, the importance of proteases in epidermal differentiation was emphasized by the discovery of null mutations of the human cathepsin C (*CTSC*) gene that result in rare palmoplantar keratoderma disorders, such as Hain-Munk and Papillon-Lefèvre syndromes.[50,51] Within the first 3 years of life, Papillon-Lefèvre syndrome patients show severe periodontitis resulting in premature tooth loss and palmoplantar keratosis, varying from mild psoriasiform skin to manifest hyperkeratosis. Most Papillon-Lefèvre syndrome patients display both periodontitis and hyperkeratosis, but some of them have only palmoplantar keratosis or periodontitis. However, *Ctsc−/−* mice develop normally and do not exhibit periodontal disease or hyperkeratosis, at least under pathogen-free conditions. Nevertheless, their cytotoxic T lymphocytes and lymphokine-activated killer cells lack cytotoxic activity because of defective processing and activation of granzymes A and B.[52] Interestingly, the *nkt* mice maintained under non-SPF conditions display some characteristics of Papillon-Lefèvre syndrome human patients, such as epidermal hyperkeratosis and dental disease, albeit with later onset. It remains to be elucidated whether, in the mouse, CTSL has complementary functions with CTSC.

Although allelic variants of the *Ctsl* gene, some aspects of the skin phenotype of *nkt* mice differ from the descriptions using *Ctsl−/−* and *fs/fs* mice. For instance, the marked hyperplasia of the sebaceous glands is an early event (day 15 postpartum) in the *nkt* mice and has not been described in the other *Ctsl*-deficient mouse models. The human skin disease nevus sebaceus of Jadassohn exhibit massive development of sebaceous glands, with epidermal, follicular, and apocrine gland abnormalities.[53] The only animal model for nevus sebaceus of Jadassohn was described by Prouty and colleagues[54] using a nude mouse grafting model. Thus, the *nkt* mutation may provide a useful model for the study of human diseases associated with sebaceous gland hyperplasia.

In conclusion, we have described in this study the skin phenotype of the *nkt* mouse mutation. The *nkt* mutation represents a potential animal model to investigate the biochemical mechanisms involved in human diseases with abnormal HFs, epidermis, and sebaceous glands. This is sustained by the availability of the mutant allele in well-defined full-congenic strains maintained in SPF conditions. In addition, we excluded the immunological factors as a potential cause of the *nkt* skin phenotype by crossbreeding *in vivo* experiments using T- and B-cell-deficient *Rag2* null mice with *nkt/nkt* mice. Finally, the data reported here provide further evidence *in vivo* that the lysosomal cysteine protease CTSL plays a critical role in HF morphogenesis, cycling, and epidermal differentiation, and that CTSL is required, but not essential, for an efficient degradation of profilaggrin and filaggrin.

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